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Identification of a Gene Involved in Assembly of *Actinomyces naeslundii* T14V Type 2 Fimbriae

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The nucleotide sequence of the *Actinomyces naeslundii* T14V type 2 fimbrial structural subunit gene, *fimA*, and the 3′ flanking DNA region was determined. The *fimA* gene encoded a 535-amino-acid precursor subunit protein (FimA) which included both N-terminal leader and C-terminal cell wall sorting sequences. A second gene, designated *orf365*, that encoded a 365-amino-acid protein which contained a putative transmembrane segment was identified immediately 3′ to *fimA*. Mutants in which either *fimA* or *orf365* was replaced with a kanamycin resistance gene did not participate in type 2 fimbriae-mediated coaggregation with *Streptococcus oralis* 34. Type 2 fimbrial antigen was not detected in cell extracts of the *fimA* mutant by Western blotting with anti-*A. naeslundii* type 2 fimbrial antibody, but the subunit protein was detected in extracts of the *orf365* mutant. The subunit protein detected in this mutant also was immunostained by an antibody raised against a synthetic peptide representing the C-terminal 20 amino acid residues of the predicted FimA. The antipeptide antibody reacted with FimA isolated from the recombinant *Escherichia coli* clone containing *fimA* but did not react with purified type 2 fimbriae in extracts of the wild-type strain. These results indicate that synthesis of type 2 fimbriae in *A. naeslundii* T14V may involve posttranslational cleavage of both the N-terminal and C-terminal peptides of the precursor subunit and also the expression of *orf365*.

Two major fimbrial types have been identified in strains of *Actinomyces naeslundii* that colonize the oral cavity. Fimbriae designated type 1 mediate bacterial adherence to salivary proline-rich proteins that coat the tooth enamel (9, 18). In contrast, those designated type 2 exhibit a lectin activity (3) that was initially detected by the lactose-sensitive coaggregation of *A. naeslundii* strains with several streptococcal strains, such as *Streptococcus oralis* 34 (27), that also colonize teeth. Type 2 fimbriae also mediate bacterial adhesion to various host cells (3), including erythrocytes, epithelial cells, and polymorphonuclear leukocytes. Activation of the latter cell type by type 2 fimbriated *Actinomyces* strains results in phagocytosis and bacterial killing (32) and the release of mediators such as superoxide (33) that may contribute to the initiation of gingival inflammation. Consequently, the identification of the fimbrial lectin(s) would provide an improved understanding of bacterium-host cell interactions. However, the nature of the type 2 fimbria-associated lectin activity, whether it is a part of the major fimbrial subunit or a minor fimbrial component, remains unknown. A major obstacle in distinguishing between these alternatives is the inability to dissociate *A. naeslundii* fimbriae to monomer subunits.

The lectin-like adhesins of several gram-negative bacteria have been identified in studies of fimbria biogenesis at the genetic level (22, 39). However, little is known concerning bacterial adhesins and assembly of fimbriae in gram-positive bacteria. The expression of both type 1 and type 2 fimbriae by *A. naeslundii* T14V (8) makes this strain a model system for studies of biogenesis of fimbriae in gram-positive bacteria.

The genes that encode the structural subunits of *A. naeslundii* T14V type 1 and type 2 fimbriae and *A. naeslundii* WVU45 type 2 fimbriae have been cloned previously, and results indicate that these genes encode proteins of approximately 54 to 59 kDa (13, 45–47). Nucleotide sequencing of the type 1 subunit of strain T14V and the type 2 subunit of strain WVU45 (47) revealed significant similarity between the encoded proteins. These studies also showed the presence in each subunit of an N-terminal leader and a C-terminal cell wall sorting signal, which is common among gram-positive cell surface proteins (37). The detection of a cell wall sorting signal in the fimbrial subunits is of interest since individual subunits are not expected to become covalently anchored to the cell wall peptidoglycan. The possible role of this sorting signal in fimbrial processing and polymerization in *A. naeslundii* has not been examined. Interestingly, results from a recent study showed that mutant strains generated by insertional inactivation of a fimbria-associated gene, *orf4*, 3′ to the *A. naeslundii* T14V type 1 fimbrial subunit gene expressed subunits that were not assembled into functional type 1 fimbriae (49). A comparison of unassembled to polymerized subunits would provide insights into assembly of fimbriae.

The *A. naeslundii* T14V type 2 fimbrial subunit gene, *fimA*, was cloned and expressed previously in *Escherichia coli* from a recombinant cosmid, pAV1402 (13). This clone expressed a protein of approximately 59 kDa that was detected with an antibody raised against type 2 fimbriae (5). In this report, we present the nucleotide sequence of *fimA* and an additional gene, designated *orf365*, 3′ to *fimA*. Mutants generated by allelic replacement of either *fimA* or *orf365* were examined for type 2 fimbria expression and fimbria-mediated adherence. The immunoreactions of fimbrial antigens from wild-type and isogenic mutants were compared with those of antibodies against either type 2 fimbriae from *A. naeslundii* T14V or a 20-amino-acid synthetic peptide prepared from the predicted C-terminal sequence of the fimbrial subunit. The results demonstrate clearly that expression of both *fimA* and *orf365* was required for the synthesis of type 2 fimbriae. Moreover, the

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TABLE 1. Bacterial strains and plasmids used

Bacterial strain or plasmid	Relevant phenotype and/or genotype[a]	Reference or source
Strain		
A. naeslundii		
T14V[b]	Wild-type strain, expresses type 1 and 2 fimbriae, Sm^r Km^s	8
5951	Spontaneous mutant of strain T14V, lacks type 1 fimbriae, expresses type 2 fimbriae, Sm^r Km^s	8
147	Spontaneous mutant of strain 5951, lacks type 1 and 2 fimbriae, Sm^r Km^s	8
MYT2-DC7[c]	Lacks type 2 fimbriae, Δ*fimA*::*kan* Sm^r Km^r	This study
MYT2-SC8[d]	Lacks type 2 fimbriae, Δ*fimA*::*kan*, contains a copy of pMY2201, Sm^r Km^r	This study
MYT2-SC3[d]	Expresses FimA, contains a copy of pMY2201, Δ*fimA*::*kan* Sm^r Km^r	This study
MY2366-DC2[c]	Expresses FimA, Δ*orf365*::*kan* Sm^r Km^r	This study
MY2366-SC1[d]	Expresses type 2 fimbriae, Δ*orf365*::*kan*, contains a copy of pMY2366, Sm^r Km^r	This study
S. oralis 34	Synthesizes cell wall polysaccharide that serves as the receptor for *Actinomyces* type 2 fimbriae	26, 27
E. coli		
DH5α	Ap^s	Gibco-BRL
TG1	Ap^s	Amersham
JM109	Ap^s	Gibco-BRL
AV3502	TG1 carrying pGP1-2 and pAV3502, Ap^r Km^r	This study
Plasmids		
pAV1402	Contains a 48-kb *A. naeslundii* T14V chromosomal DNA subcloned onto pHC79	13
pAV3022	Contains a 9.0-kb *Hin*dIII DNA fragment from pAV1402 subcloned onto pUC13, Ap^r	This study
pAV3502	Contains a 2.4-kb *Sma*I DNA fragment from pAV3022 subcloned onto pGEM3Z for high-level expression of FimA, Ap^r	This study
pAV2606	Contains a 1.85-kb *Bam*HI DNA fragment from pAV3022 that encoded a 22-kDa truncated amino-terminal portion of FimA, Ap^r	This study
pAV2621	Contains a 4.4-kb *Bam*HI DNA fragment from pAV3022 that encoded a 35-kDa truncated carboxyl-terminal portion of FimA, Ap^r	This study
pMY300	Contains the *kan* gene from pJRD215 subcloned onto pGEM7Zf(+), Ap^r Km^r	This study
pMY221	Contains a 2.4-kb *Sma*I DNA fragment from pAV3022 subcloned onto pUC13 that harbors *A. naeslundii* T14V *fimA*, Ap^r	This study
pMY2201	The 975-bp *Kpn*I DNA fragment internal to *fimA* is deleted and replaced with the *kan* gene from pJRD215; transcription of *kan* is opposite that of *fimA*; Δ*fimA*::*kan* Ap^r Km^r	This study
pMY2366	The 600-bp *Bst*XI DNA fragment internal to *orf365* is substituted with the *kan* gene; transcription of *kan* is opposite that of *orf365*; Δ*orf365*::*kan* Ap^r Km^r	This study
pJRD215	Km^r Sm^r	10
pUC13	Ap^r	Gibco-BRL
pGEM7Zf(+)	Ap^r	Promega
pGEM3Z	Ap^r	Promega
pGP1-2	Contains the T7 RNA polymerase gene, Km^r	40

[a] Km, kanamycin; Sm, streptomycin; Ap, ampicillin.
[b] Made resistant to streptomycin by selection of colonies from a growth medium containing the antibiotic.
[c] Mutant strain generated by a double-crossover recombinational event.
[d] Mutant strain generated by a single-crossover recombinational event predicted by the mechanism of Campbell.

carboxyl-terminal peptide of the precursor fimbrial subunit appeared to have been cleaved during assembly. To our knowledge, the proposed posttranslational modification is a novel step in biogenesis of fimbriae.

MATERIALS AND METHODS

Bacteria and plasmids. Bacterial strains and plasmids used in this study are described in Table 1. A complex medium (7) or *Lactobacillus*-carrying medium (15) supplemented with 20 mM D,L-threonine was used to prepare cultures of *Actinomyces* strains, and Luria-Bertani (LB) (31) was used for *E. coli* strains. The antibiotics (Sigma Chemical Co., St. Louis, Mo.) used in this study were kanamycin sulfate, streptomycin, and ampicillin, at 40, 50, and 100 μg/ml, respectively.

Preparation of *A. naeslundii* T14V type 2 fimbrial antigens. Type 2 fimbriae were isolated from *A. naeslundii* 5951, a spontaneous mutant that expresses only type 2 fimbriae (Table 1). Bacteria from the stationary phase of growth were washed with Tris HCl-buffered saline (TBS; 0.15 M NaCl, 0.02 M Tris-HCl [pH 7.8], 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.02% sodium azide) and subjected to sonication (4). Fimbriae collected in fractions at or near the void volume of a Sephacryl S400 (Pharmacia Biotech, Inc., Piscataway, N.J.) column were purified by fractional ammonium sulfate precipitation at 4°C as described previously (5, 29). Edman degradation of purified type 2 fimbriae was performed as described previously (47).

The precursor subunit protein (FimA) from *E. coli* AV3502 was purified by a procedure similar to that described previously (45). A sonicated extract was applied to a DEAE-Sephacel (Pharmacia Biotech) column and eluted with a gradient of 0.05 to 0.2 M NaCl in TBS. FimA was monitored by solid-phase immunoassay with anti-*A. naeslundii* T14V type 2 fimbrial antibody and further purified by Sephacryl S-300 (Pharmacia Biotech) gel filtration column chromatography. Final purification of FimA was by immunoaffinity chromatography with a column prepared with an anti-*A. naeslundii* T14V type 2 fimbrial monoclonal antibody and by elution with 3 M sodium thiocyanate. The concentration of antigens was determined by a micro-bicinchoninic acid protein assay (Pierce, Rockford, Il.), using bovine serum albumin as the standard.

A synthetic peptide consisting of an amino-terminal cysteine followed by the carboxyl-terminal 20 amino acid residues (VGSVLVARYRERKQNANLAL) of *A. naeslundii* T14V FimA was synthesized on a 430A automated peptide synthesizer (Applied Biosystems, Inc., Foster City, Calif.). The amino acid composition

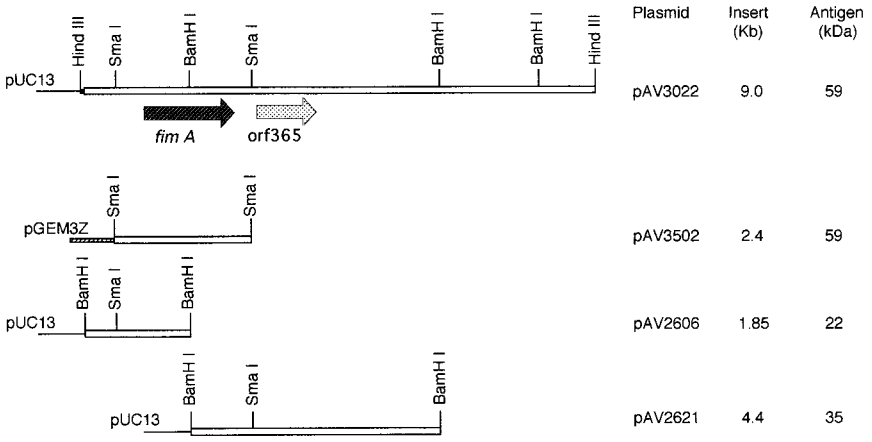

FIG. 1. Restriction endonuclease maps of recombinant cosmid pAV1402 and its derivatives. The size of the inserted *A. naeslundii* T14V DNA in each plasmid and the apparent molecular weights of the plasmid-encoded proteins detected by immunostaining with the anti-*A. naeslundii* T14V type 2 fimbriae antibody are indicated. Selected restriction endonuclease recognition sites are included for reference. Symbols: ■, pHC79 DNA; ——, pUC13 DNA; ▨, pGEM3Z DNA; and □, *A. naeslundii* T14V DNA.

of the peptide was determined by amino acid analysis as described previously (47). For immunization, the peptide was conjugated to keyhole limpet hemocyanin (KLH), using sulfo-*m*-maleimidobenzoyl-*N*-hydroxysulfo-succinimide ester (Pierce) as the cross-linker.

Antisera and immunological methods. A rabbit was immunized with peptide-KLH conjugate (1 mg) in Freund's complete adjuvant on day 1 and the same amount of conjugate in incomplete adjuvant on days 21, 42, and 63. The anti-peptide antiserum (JC8) was obtained 1 week after the last injection. The production of rabbit antiserum (R55) against purified *A. naeslundii* T14V type 2 fimbriae and the preparation of monospecific immunoglobulin G (IgG) fractions from these antisera have been described previously (5, 9).

Enzyme-linked immunosorbent assay (ELISA) was performed with flat-bottom wells of Immulon I plates (Dynatech Laboratories, Inc., Alexandria, Va.) that were coated overnight at 4°C with purified type 2 fimbriae (3 μg/ml), purified recombinant subunit (3 μg/ml), or synthetic peptide (100 μg/ml). The amount of antibody bound to adsorbed antigen was detected with a secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories, Hercules, Calif.). Western blotting was performed as described previously (44), using either rabbit immune IgG (1 μg/ml) or antiserum diluted to at least 1:500. Horseradish peroxidase-conjugated goat anti-rabbit IgG was the secondary antibody, and blots were developed with reagents supplied in the immunoassay kit (Bio-Rad Laboratories).

Molecular DNA manipulations. Restriction endonuclease maps of plasmid DNA were determined by standard methods (31). Subcloning and construction of integration vectors were performed with DNA fragments eluted from agarose gels with reagents from an Elu Quik kit (Schleicher & Schuell, Keene, N.H.). Ligations and transformations of *E. coli* with various constructs were performed by procedures described previously (14, 31). The host strain for plasmids pAV3022, pAV2621, and pAV2606 was *E. coli* JM109. For plasmids pMY221, pMY2366, and pMY2201, *E. coli* DH5α was the host strain, and *E. coli* TG1 carrying the resident plasmid pGP1-2 (Amersham Life Science Inc., Arlington Heights, Ill.) (40) was the host strain for plasmid pAV3502. Transformants were selected on LB agar containing antibiotics, and plasmid DNA was isolated by the alkaline lysis method and purified by CsCl-ethidium bromide density gradient centrifugation (31). The nucleotide sequence of *A. naeslundii* T14V chromosomal DNA in plasmids pAV3502 and pAV2621 was determined by the Sanger dideoxy-chain termination procedure (34), using a Sequenase kit (version 7.0; United States Biochemical Corp., Cleveland, Ohio) and [^{35}S]dATP (12.5 mCi/ml; DuPont England Nuclear, Boston, Mass.). Primers for DNA sequencing and for amplification of DNA fragments by PCR were prepared on an Applied Biosystems model 391 DNA synthesizer. PCR was performed with either *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) or *Taq* DNA polymerase (Life Technologies, Inc.), using conditions similar to those described previously (44). Nucleotide sequences were analyzed by using the software package of the Genetics Computer Group (version 9.0; University of Wisconsin Biotechnology Center) (12). Sequence homology searches to other bacterial proteins in public databases were performed with the program BLAST (1).

Isolation and characterization of mutants. Purified integration plasmid DNA (100 ng) was used to transform *A. naeslundii* T14V by electroporation (48), and transformants were selected on brain heart infusion agar containing kanamycin and streptomycin. Chromosomal DNA from mutants was digested with restriction endonucleases, separated by agarose gel electrophoresis, and analyzed by Southern blot hybridization, under conditions of high stringency, to various ^{32}P-labeled DNA probes (43). Briefly, DNA on filters was prehybridized at 42°C for 2 to 4 h in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% formamide, 10% dextran sulfate (Pharmacia Biotech), 1× Denhardt's solution, 1% sodium dodecyl sulfate (SDS), 1 M NaCl, 0.5% sodium pyrosphosphate, and 200 μg of denatured herring sperm DNA (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml. Hybridizations were at the same temperature for 18 to 20 h. Posthybridization washes were with 0.1× SSC–0.5% SDS at 65°C for 1 h with one change of buffer. Sonicated bacterial cell extracts from *A. naeslundii* strains were prepared as described previously (44). A cell extract enriched for cytoplasmic proteins also was obtained by disruption of washed bacteria which had been passed twice at 500 lb/in^2 (equivalent to cell pressure of 8,000 lb/in^2) through a French pressure cell (American Instrument Co., Silver Spring, Md.). Cell debris was removed by centrifugation at 16,000 rpm in a SW40Ti rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 1 h at 4°C, and the supernatant fluid was brought to 40% saturation with solid ammonium sulfate at 4°C. The precipitated proteins were dissolved in TBS and dialyzed extensively against TBS prior to SDS-polyacrylamide gel electrophoresis (PAGE) (25) on 10% polyacrylamide gels and transfer to nitrocellulose.

The adherence properties of each mutant strain were assessed by the coaggregation assay using *S. oralis* 34 as the partner strain (26). Cell suspensions (5×10^8 in a final volume of 50 μl) of *A. naeslundii* parent or mutant strains and *S. oralis* 34 were mixed in wells of microtiter plates, and results were scored as described previously (7). Reversibility of coaggregation was determined in the presence of 125 mM lactose.

Nucleotide sequence accession number. The nucleotide sequence reported in this study was assigned GenBank accession no. AF019629.

RESULTS

Restriction site mapping, subcloning, and expression. A restriction endonuclease map was determined for the recombinant cosmid pAV1402 (13), and *fimA* was localized on the inserted DNA fragment by immunological screening of various subclones with anti-*A. naeslundii* T14V type 2 fimbrial antibody. The 59-kDa type 2 fimbrial subunit protein was detected by Western blot analysis in a subclone carrying the plasmid pAV3022, which contained a 9.0-kb *Hin*dIII DNA fragment from pAV1402 (Fig. 1). Shotgun subcloning of pAV3022 after *Sma*I digestion resulted in the isolation of plasmids pAV3502 (Fig. 1) and pMY221 (Table 1), which had an insertion of a 2.4-kb *Sma*I DNA fragment that encoded the 59-kDa subunit protein. Two additional derivatives, designated pAV2606 and pAV2621, which contained *Bam*HI DNA fragments from pAV3022 were obtained. These subclones encoded proteins of approximately 22 and 35 kDa, respectively, that were immunostained with anti-*A. naeslundii* T14V type 2 fimbrial antibody. From the physical maps of the plasmids, these proteins represented the truncated N- and C-terminal portions, respectively, of the structural subunit (Fig. 1). Expression of these

truncated proteins was directed by the promoter of the vector, and the *A. naeslundii* T14V DNA in pAV2621 was fused in frame to the *lacZ′* sequence of pUC13.

Sequence analysis of *fimA* and a putative gene 3′ to *fimA*. The nucleotide sequences of the 2.4-kb *Sma*I and the 4.4-kb *Bam*HI DNA fragments from pAV3502 and pAV2621, respectively, were determined. The DNA sequence containing *fimA* and an open reading frame, designated *orf365*, is presented in Fig. 2. Both *fimA* and *orf365* started with an ATG initiation codon that was preceded by a putative ribosomal binding site (Fig. 2) (38). The *fimA* gene (nucleotides 482 to 2086) encoded a predicted protein of 535 amino acids, and the amino-terminal 30+ residues of the deduced protein had properties characteristic of a leader sequence (41). Results of Edman degradation of type 2 fimbriae purified from *A. naeslundii* T14V identified glutamate at position 34 of the predicted protein sequence as the N-terminal amino acid. The next 29 amino acid residues determined by amino acid sequencing were identical to the predicted protein sequence (Fig. 2). Thus, the putative leader sequence cleavage site of the precursor protein encoded by *fimA* is between threonine and glutamate at positions 33 and 34, respectively (Fig. 2).

The predicted type 2 fimbrial subunit of *A. naeslundii* T14V, obtained following cleavage of the leader, consisted of 502 amino acid residues and had a calculated molecular weight of 52,847. The hydropathy of the predicted protein, plotted by the Kyte-Doolittle method (24), suggested a molecule that was predominately hydrophilic except for the presence of a hydrophobic region at the C-terminal end. The sequence of the C-terminal 43 amino acid residues resembled that of a cell wall sorting signal (28, 36, 37) which has been noted in many gram-positive cell surface proteins and other *Actinomyces* fimbrial subunits (46, 47). This signal comprises the consensus cell wall anchoring motif, LPXTG (Fig. 2), followed, in sequence, by a hydrophobic domain and a positively charged hydrophilic domain. To detect the C-terminal end of the fimbrial subunit protein, rabbit antibody was prepared against a synthetic peptide consisting of the C-terminal 20 amino acid residues (Fig. 2). This antiserum reacted strongly in ELISA with the recombinant subunit protein (FimA) purified from *E. coli* AV3502 and with the unconjugated synthetic peptide. Significantly, the antibody did not react above the level of preimmune serum with type 2 fimbriae purified from strain T14V (Table 2). In contrast, rabbit antiserum against type 2 fimbriae from strain T14V did not react above the level of preimmune serum with the unconjugated peptide but reacted strongly both with the recombinant fimbrial subunit and with type 2 fimbriae. Comparable results were obtained with purified type 2 fimbriae and recombinant subunit protein that were subjected to SDS-PAGE and transferred to nitrocellulose. The patterns from Western blotting with anti-*A. naeslundii* T14V type 2 fimbriae antibody were similar to those observed previously (13) and consisted of a characteristic ladder of high-molecular-weight proteins in addition to a relatively weak band of monomeric subunit for type 2 fimbriae and a single band at 59 kDa for the recombinant protein. In contrast, the antipeptide antibody did not detect bands from transferred fimbriae but reacted strongly with the recombinant FimA (profile not shown). Thus, epitopes associated with the C-terminal 20 amino acids predicted by the nucleotide sequence of *fimA* were detected in the recombinant fimbrial subunit but not in fimbriae isolated from *A. naeslundii* T14V cell surface.

Two inverted repeats were located immediately 3′ of the termination codon of *fimA* (nucleotides 2097 to 2134 and 2217 to 2762) (Fig. 2). The calculated free energies (17) of these potential hairpin structures were −35 and −49 kcal, respectively. A predicted RNA secondary structure encompassing these repeat sequences generated by the program FOLD (50) had an overall calculated free energy of −137.7 kcal. This region of dyad symmetry was followed by a putative gene, *orf365* (nucleotides 2468 to 3562), that encoded a predicted protein of 365 amino acids with a calculated molecular weight of 39,425. Similar to the type 2 fimbrial structural subunit, ORF365 also was predominately hydrophilic (24). However, unlike the subunit protein, no detectable leader sequence or cell wall sorting signal motifs were observed in ORF365. Further analysis of ORF365 suggested that this protein contained one membrane helix (between amino acids 238 and 255) (Fig. 2) (42). Results of a topology prediction (with a reliability of 7 on a scale of 0 to 9, with 9 being most reliable) (30) indicated that the N-terminal two-thirds and the C-terminal one-third of ORF365 were located outside and inside the cytoplasmic membrane, respectively.

Sequence homology between FimA or ORF365 and other bacterial proteins was noted only with fimbria-associated proteins from *A. naeslundii* T14V or WVU45 (46, 47, 49). Sequence alignments by the program Bestfit (Genetics Computer Group) showed significantly greater sequence similarity between FimA of strain T14V and the type 2 fimbrial subunit of strain WVU45 (65% sequence identity and 77% similarity) (Fig. 3A) (46) than between FimA and the type 1 fimbrial subunit (FimP) of strain T14V (31% sequence identity and 38% similarity) (Fig. 3B). Significant homology (40% identity and 47.5% similarity) also was noted between the amino-terminal half of ORF365 and the central region of the predicted protein encoded by *orf4*, a type 1 fimbria-associated gene of *A. naeslundii* T14V (49).

Construction of *fimA* and *orf365* mutants. Integration plasmids pMY2201 and pMY2366 were constructed by substituting the kanamycin resistance (*kan*) gene from pJRD215 (10) for the 975-bp *Kpn*I and 600-bp *Bst*XI DNA fragments internal to *fimA* and *orf365*, respectively (Table 2 and Fig. 2). Kanamycin-resistant transformants were obtained by transformation of *A. naeslundii* T14V with these plasmids. The physical maps of representative mutants (Fig. 4) were determined by Southern blot analysis of genomic DNA digested with various restriction endonucleases and hybridized to various DNA probes, including pUC13 DNA, *kan*, *fimA*, and *orf365*. Results of these analyses showed that strains MY2T-DC7 and MY2366-DC2 were generated by allelic replacement of *fimA* and *orf365*, respectively, with the *kan* gene. The lack of hybridization signal between strain MYT2-DC7 and the 975-bp *Kpn*I DNA internal to *fimA*, and between strain MY2366-DC2 and the 600-bp *Bst*XI DNA internal to *orf365*, confirmed that each specific DNA sequence was deleted from the respective mutant. As expected from the insertion-and-duplication mechanism predicted by Campbell (2), two types of single-crossover mutants were obtained with pMY2201. Those like strains MYT2-SC8 and MYT2-SC3 each contained a copy of the intact *fimA* sequence (Fig. 4). The only single-crossover mutants obtained with pMY2366 were those like MY2366-SC1 (Fig. 4); mutants with insertions of pMY2366 between the *Bam*HI site in *fimA* and the 5′ *Bst*XI site of *orf365* were not isolated.

Roles of *fimA* and *orf365* in biogenesis of fimbriae. Type 2 fimbria-mediated adherence, as determined by the lactose-sensitive coaggregation of *Actinomyces* strains with *S. oralis* 34, was completely abolished by allelic replacement of *fimA* in strain MYT2-DC7 and by integration of pMY2201 in *fimA* of strain MYT2-SC8 or MYT2-SC3. No type 2 fimbrial antigens were detected in strains MYT2-DC7 and MYT2-SC8 (Fig. 5A, lanes 2 and 4, respectively), as shown by Western blot analysis of sonicated cell extracts with anti-type 2 fimbrial antibody.

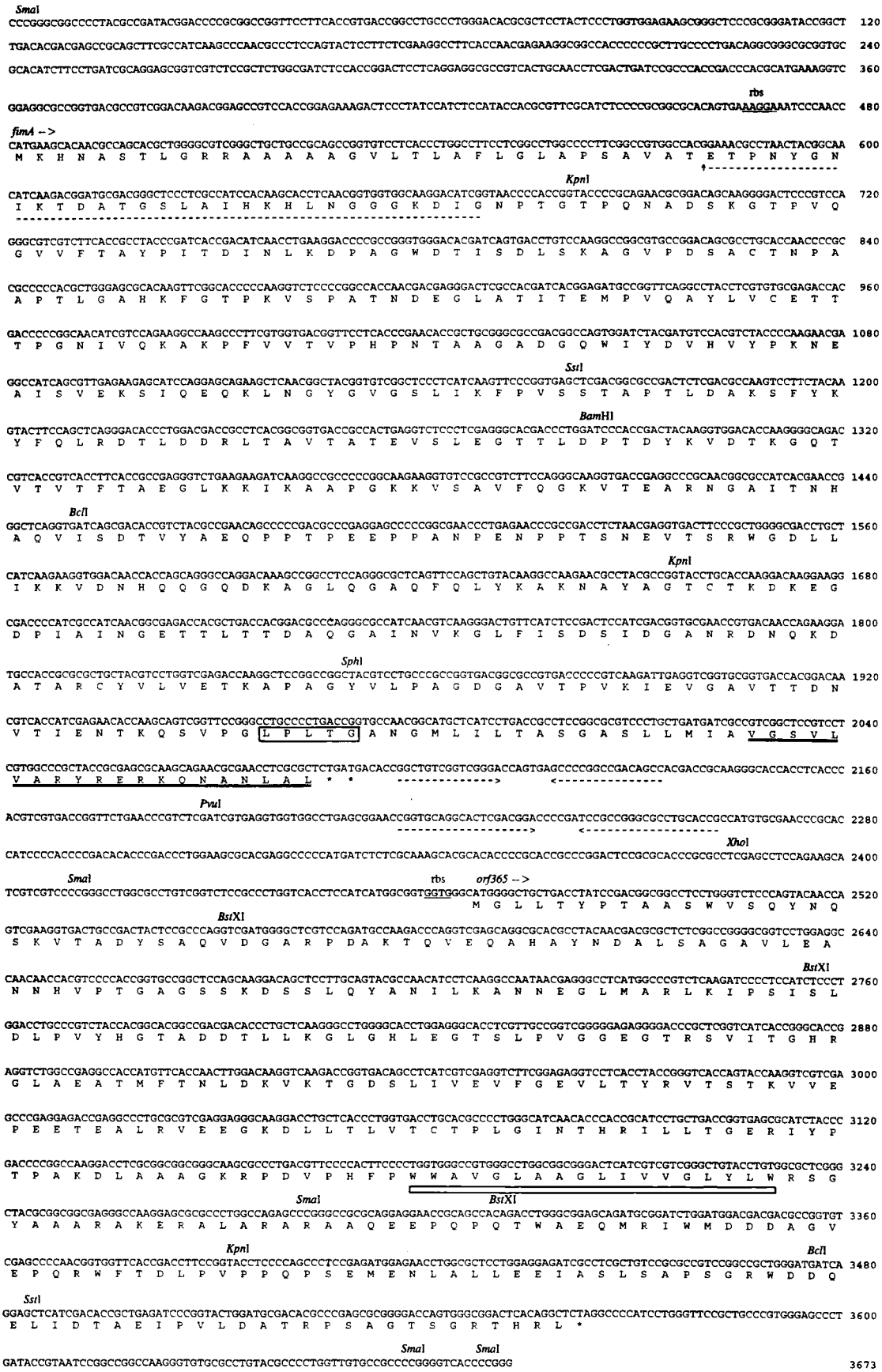

FIG. 2. Nucleotide sequence of a 3.67-kb *A. naeslundii* T14V chromosomal DNA region containing the type 2 fimbrial subunit gene, *fimA*, and a putative gene, *orf365*, involved in fimbrial biogenesis. The presumptive ribosomal binding site (rbs; underline), two inverted repeats (arrow and dotted underline) downstream of the termination codon, TGA (*), of *fimA*, the amino-terminal amino acid sequence of type 2 fimbriae (dotted underline) determined by Edman degradation, the leader peptide processing site (upward arrow) of the subunit precursor, the conserved cell wall anchoring motif (LPXTG; boxed), the 20-amino-acid carboxyl-terminal sequence (thick underline) of FimA used to prepare a rabbit antipeptide antibody, and the putative transmembrane segment in ORF365 (open bar) are indicated. Selected restriction endonuclease recognition sites are included for reference.

Thus, although strain MYT2-SC8 contained a copy of *fimA* (Fig. 4), the lack of FimA production suggested that *fimA* might be part or an operon or that the expression of genes 5′ to *fimA* was required for *fimA* expression. Some high-molecular-weight protein bands along with the fimbrial subunit were present in strain MYT2-SC3 (Fig. 5A, lane 3). However, minor differences were noted between the immunostained protein profile of this strain and that of the wild-type strain (Fig. 5A, lane 1), suggesting the possibility of truncated type 2 fimbriae produced by this strain.

Lactose-sensitive coaggregation activity was also abolished by allelic replacement of *orf365* in mutant strain MY2366-DC2 but was not affected by insertion of pMY2366 3′ to *orf365* in strain MY2366-SC1. Only the fimbrial subunit protein was detected in sonicated extract of strain MY2366-DC2 along with immunoreactive bands of lower molecular weight (Fig. 5A, lane 5). The latter bands were degradative products of the subunit, as suggested previously (13). In contrast, the immunoblot profile of sonicated extract of strain MY2366-SC1 was similar to that observed in the wild-type strain T14V or strain 5951, which expresses only type 2 fimbriae (Fig. 5A; compare lane 6 to lanes 1 and 8). The subunit expressed by strains MY2366-DC2 and MYT2-SC3 was also detected by Western blotting with the antipeptide antibody prepared against the C-terminal end of FimA. The subunit protein appeared as a sharp narrow immunostained band with this antibody but as a broadly stained band with the anti-type 2 fimbrial antibody, even though equal amounts of sonicated extract were used in both analyses. A difference in the amount of antigen detected by the antipeptide antibody in strain MY2366-DC2 and MYT2-SC3 also was indicated by the greater intensity of the subunit band observed with 35 and 70 μg, respectively, of sonicated extract from these strains (Fig. 5B, lanes 1 and 2). Significantly, the antipeptide antibody did not react with 150 μg of sonicated extracts or French press extracts from strains 147 and 5951 and wild-type strain T14V (Fig. 5B, lanes 3, 4, and 5, respectively). However, the subunit and higher-molecular-weight type 2 fimbrial antigens were readily detected by Western blotting with anti-type 2 fimbrial antibody in the extracts of strains T14V and 5951 (Fig. 5A, lanes 1 and 8, respectively). The specificity of the anti-type 2 fimbrial antibody used in these experiments was indicated by the absence of any reaction with strain 147, which lacks both type 1 and 2 fimbriae (Fig. 5A, lane 7; Fig. 5B, lane 3). Thus, the unassembled subunit expressed in the mutant strain MY2366-DC2 or MYT2-SC3 also contained the C-terminal peptide of the precursor subunit FimA.

DISCUSSION

Results from this study demonstrate that at least two genes, namely, the fimbrial subunit gene, *fimA*, and the 3′ adjacent gene *orf365*, are necessary for the synthesis of functional type 2 fimbriae in *A. naeslundii* T14V. The conclusion is supported by the lack of expression of type 2 fimbrial antigen by the *fimA* mutant MYT2-DC7 and of assembled fimbriae by the *orf365* mutant MY2366-DC2. The mutant strains, MYT2-DC7 and MYT2-SC8, that lacked type 2 fimbriae were unable to coaggregate with *S. oralis* 34, which has a receptor polysaccharide for the type 2 fimbrial lectin (26). However, expression of *fimA* alone was not sufficient for the adherence properties observed in the wild-type strain, since mutant strains MYT2-SC3 and MY2366-DC2, which synthesized only the fimbrial subunit, also failed to coaggregate with *S. oralis* 34. In a previous study of strain T14V type 1 fimbriae (44, 49), mutants that produced the unassembled type 1 subunits but not type 1 fimbriae did not adhere to proline-rich proteins that are specific receptors of these fimbriae (18). Further studies to identify and characterize the genes involved in biogenesis of type 1 and type 2 fimbriae should provide a firm basis for associating the receptor binding sites of these structures either with the structural subunits, FimP and FimA, respectively, or with minor fimbrial proteins.

The hypothesis that *orf365* is involved in the assembly of type 2 fimbriae was supported by the following observations. First, mutants generated by allelic replacement of *orf365* coding sequence with the *kan* cassette expressed only monomeric fimbrial subunit. Second, significant sequence similarity was observed between the predicted protein encoded by *orf365* and that encoded by *orf4*, a gene which is located immediately 3′ to the *A. naeslundii* T14V type 1 fimbrial subunit gene, *fimP* (49). Third, an isogenic mutant of *orf4* created by allelic replacement also expressed only unassembled fimbrial subunits (49). These similarities suggested that *orf365* and *orf4* may play similar roles in the synthesis of type 2 and type 1 fimbriae, respectively. It is of interest that the mutant strain, MY2366-SC1, which contained pMY2366 integrated beyond the 3′ end of *orf365* produced functional type 2 fimbriae. Thus, *orf365* may be the last member of the type 2 fimbrial gene cluster(s). Indeed, analysis of the nucleotide sequence of the chromosomal DNA region between the *Sma*I site 3′ to *orf365* and the downstream *Bam*HI site (Fig. 5; note restriction endonuclease map of strain T14V [Fig. 4]) revealed the presence of genes encoding ribosomal proteins (data not shown). Further analysis of the DNA sequence 5′ of *fimA* may reveal additional fimbria-associated genes, as is the case with the type 1 fimbrial gene cluster in *A. naeslundii* T14V (49), which consists of seven genes, including *fimP*.

Striking similarities have been observed among different fimbrial types from *E. coli* and related gram-negative bacteria

TABLE 2. Reactions of rabbit antisera with *A. naeslundii* T14V type 2 fimbriae and related antigens measured by ELISA

Antigen	Dilution^{-1} of antiserum for optical density of 0.5 in ELISA	
	Anti-carboxyl-terminal peptide	Anti-*A. naeslundii* T14V type 2 fimbriae
C-terminal peptide[a]	6×10^4	$<1 \times 10^2$
FimA[b]	2×10^4	5×10^5
Fimbriae[c]	$<1 \times 10^2$	1×10^6

[a] Synthetic peptide representing the 20 amino acids at the carboxyl terminus of the *fimA*-encoded protein.
[b] Type 2 subunit protein purified from *E. coli* AV3502 (FimA).
[c] Type 2 fimbriae purified from *A. naeslundii* 5951 (fimbriae).

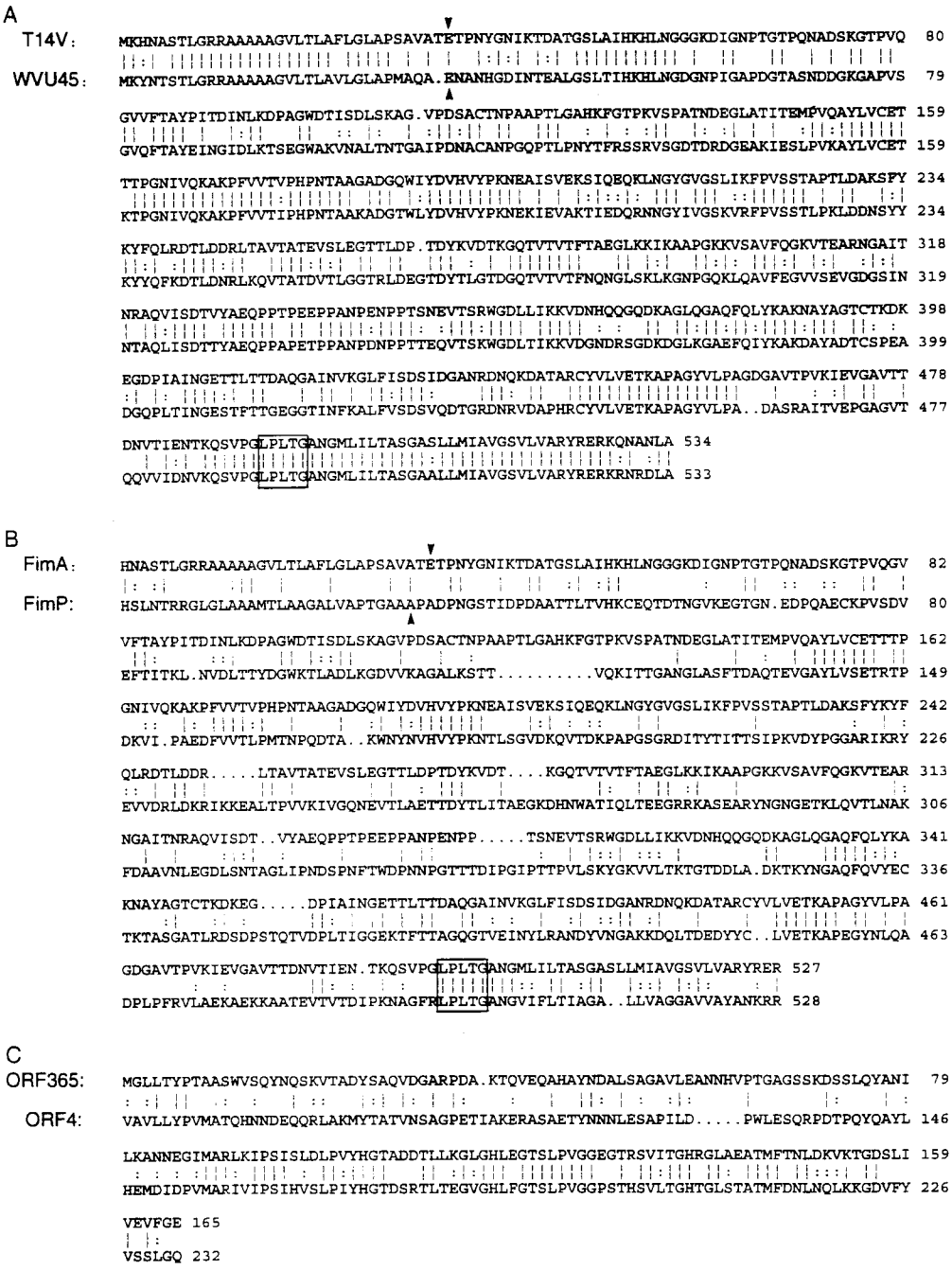

FIG. 3. Sequence homology between the deduced amino acid sequences of type 2 fimbrial subunits of *A. naeslundii* T14V and *A. naeslundii* WVU45 (A), type 2 (FimA) and type 1 (FimP) fimbrial subunits of *A. naeslundii* T14V (B), and the N-terminal portion of the protein encoded by *orf365* flanking *fimA* and the central portion of the protein encoded by *orf4* flanking *fimP* of the type 1 fimbrial gene cluster (C). Identical (|) and conserved substituted (:) amino acid residues are indicated. The N-terminal amino acid (downward arrow) determined by Edman degradation of purified fimbriae and the consensus cell wall anchoring motif (LPXTG; boxed) are indicated.

(11, 16, 19, 23, 39). The similarities include significant homologies between structural subunit and fimbria-associated proteins. In addition, the organizations of genes that encode major and minor fimbrial components, chaperone proteins, or other proteins involved in subunit transport and control of fimbrial assembly are similar (11, 16, 19, 23). Information gained from the sequence analysis of *fimA* and its gene product in this study extends previous observations that common characteristics also exist among various *A. naeslundii* fimbrial genes. Thus, each of three fimbrial subunit genes (type 1 of *A. naeslundii* T14V and type 2 of strains T14V and WVU45) encodes a precursor subunit that has a typical leader sequence of approximately 30 amino acids. The fimbrial subunits of *A. naeslundii* are generally more hydrophilic than the subunits of gram-negative bacterial fimbriae (21). At the protein level, significant sequence homology (33%) was observed between the type 1 fimbrial subunit of *A. naeslundii* T14V and the type 2 fimbrial subunit of strain WVU45 (47). A similar level of sequence

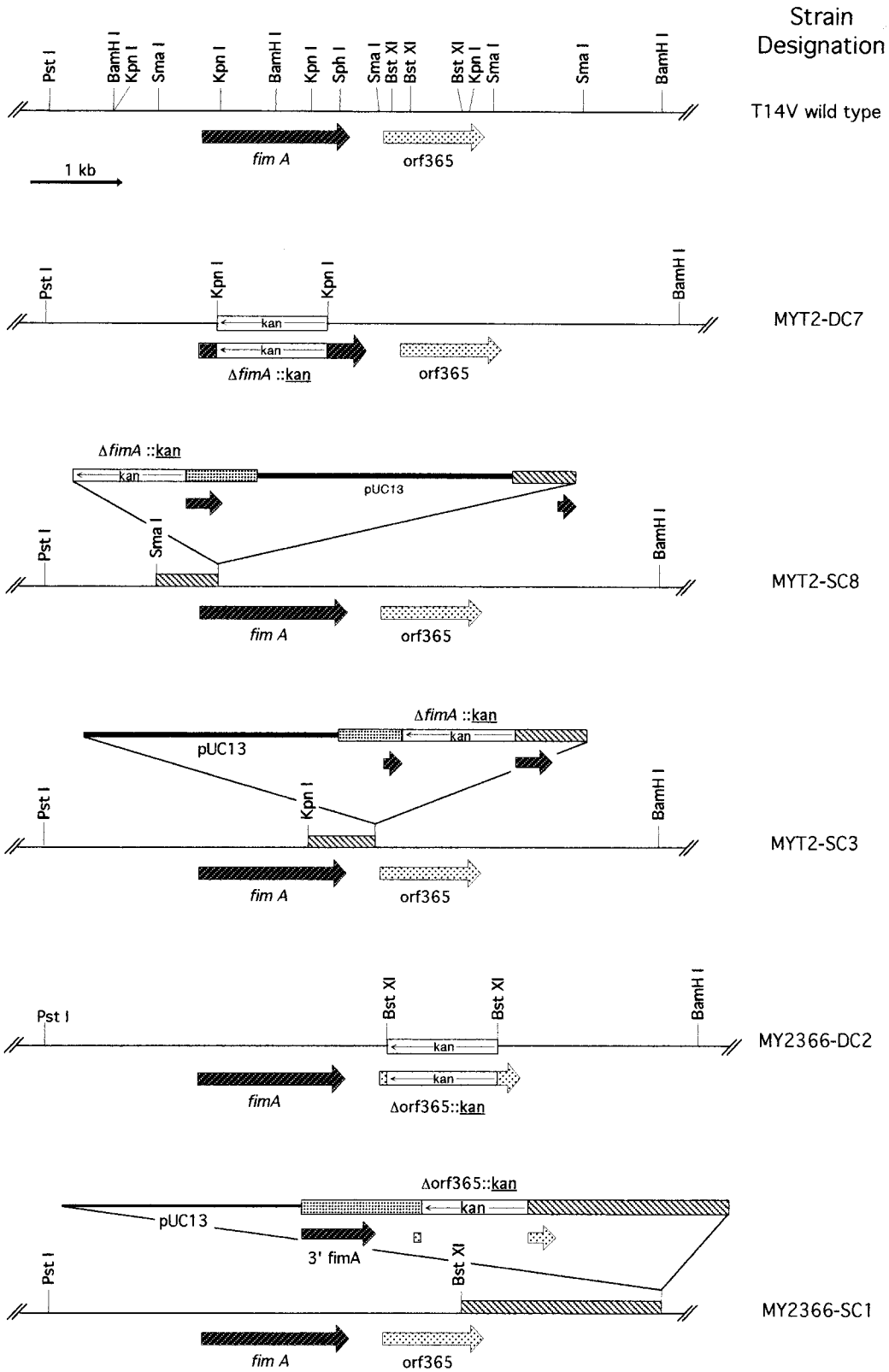

FIG. 4. Restriction endonuclease maps of *A. naeslundii* wild-type strain T14V and isogenic mutants generated by allelic replacement of *fimA* (strain MYT2-DC7) or *orf365* (strain MY2366-DC2) and those generated by single crossover with the integration plasmid pMY2201 (strains MYT2-SC8 and MYT2-SC3) or pMY2366 (strain MY2366-SC1). Only the chromosomal DNA region flanking *fimA* and *orf365* and selected restriction endonucleases are included. Symbols: ——, *A. naeslundii* T14V DNA; —, pUC13 DNA; ▭, *kan* gene; ▨, *fimA*; ▩, *orf365*. The chromosomal region where plasmid integration occurred as mediated by the Campbell insertion-duplication mechanism is also indicated (▧).

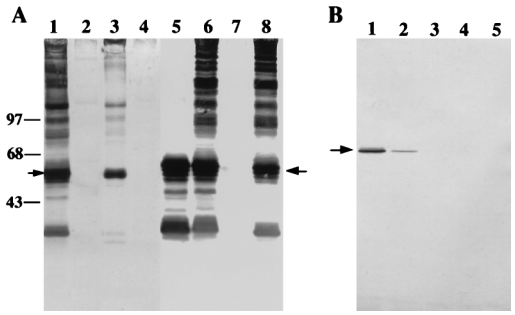

FIG. 5. (A) Composite of Western blots of sonicated cell extracts of *A. naeslundii* T14V (wild type), mutant strains MYT2-DC7, MYT2-SC3, MYT2-SC8, MY2366-DC2, and MY2366-SC1, strain 147, and strain 5951 with anti-*A. naeslundii* T14V type 2 fimbrial antibody (lanes 1 through 8, respectively). Proteins were separated by SDS-PAGE, and transferred proteins on nitrocellulose were immunostained with anti-*A. naeslundii* T14V type 2 fimbrial antibody. The apparent molecular sizes (in kilodaltons) are indicated on the left. (B) Western blot of cell extracts of *A. naeslundii* mutant strains MY2366-DC2, MYT2-SC3, 147, and 5951 and wild-type strain T14V (lanes 1 through 5, respectively). Transferred proteins on nitrocellulose were immunostained with rabbit anti-peptide antibody prepared against the predicted C-terminal sequence of FimA. Arrows indicate the 59-kDa type 2 fimbrial subunit.

homology also was observed between the *fimP*- and *fimA*-encoded proteins (Fig. 3B), suggesting that an overall sequence identity of approximately 30% may be expected between the structural subunits of *A. naeslundii* type 1 and type 2 fimbriae. Greater homology would be anticipated between the subunits of functionally similar fimbriae. Indeed, an overall sequence similarity of 77% was found between the *A. naeslundii* T14V and WVU45 type 2 fimbrial subunits (Fig. 3A) even though the type 2 fimbriae of these strains are only weakly cross-reactive (6). Finally, the relative locations of *orf365* and *orf4* with respect to the structural subunit genes would favor the hypothesis that the type 1 and type 2 fimbrial gene clusters are organized similarly.

An epitope(s) associated with the predicted carboxyl terminus of the type 2 fimbrial subunit was detected in recombinant FimA from *E. coli* and unassembled FimA synthesized by the *orf365* knockout mutant but not in fimbriae from *A. naeslundii* T14V. Thus, the C terminus of FimA must either become inaccessible to antibody or, alternatively, be removed during assembly of fimbriae. The latter possibility is favored by the results of Western blotting of cell extracts of strains 5951 and T14V in which FimA monomer was not detected by antibody against the predicted C-terminal peptide of FimA (Fig. 5B, lanes 4 and 5, respectively) but was readily detected by antibody against type 2 fimbriae (Fig. 5A, lanes 8 and 1, respectively). In addition to these findings, the removal of the peptide at the C-terminal end of FimA during assembly of fimbriae would be consistent with the presence of a cell wall sorting signal in the subunit (Fig. 2). Based on the general model for trafficking of surface proteins in gram-positive bacteria (28, 35), it is likely that the carboxyl terminus of the precursor fimbrial subunit may be cleaved between threonine and glycine of the LPLTG sequence and that the C-terminal threonine, instead of being anchored to cell wall peptidoglycan, is linked to another subunit either directly or through a peptidoglycan fragment that has yet to be detected in mature fimbriae. Alternatively, the processed subunit may be transiently associated with a cell wall protein that functions to initiate subunit assembly. In this regard, a cell-bound nucleator protein that primes the polymerization of *E. coli* curlins during pilus assembly has been described (20). The possible existence of covalent linkages between subunits of *A. naeslundii* fimbriae would account for the inability of techniques such as SDS-PAGE to dissociate these structures to subunits (3). Consistent with the predicted primary sequence and experimental data, the *A. naeslundii* fimbrial subunit precursor would be expected to undergo two posttranslational modifications: removal of the amino-terminal leader sequence during export of the precursor through the cytoplasmic membrane, and removal of the carboxyl-terminal peptide at the cell wall anchoring sequence during subunit assembly. However, the possibility that carboxyl-terminal peptide processing and fimbrial assembly are independent events cannot be excluded. Clearly, further studies are needed to define the mechanism(s) of fimbrial subunit polymerization. The results of such studies should advance our knowledge of fimbrial biosynthesis in this gram-positive species.

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